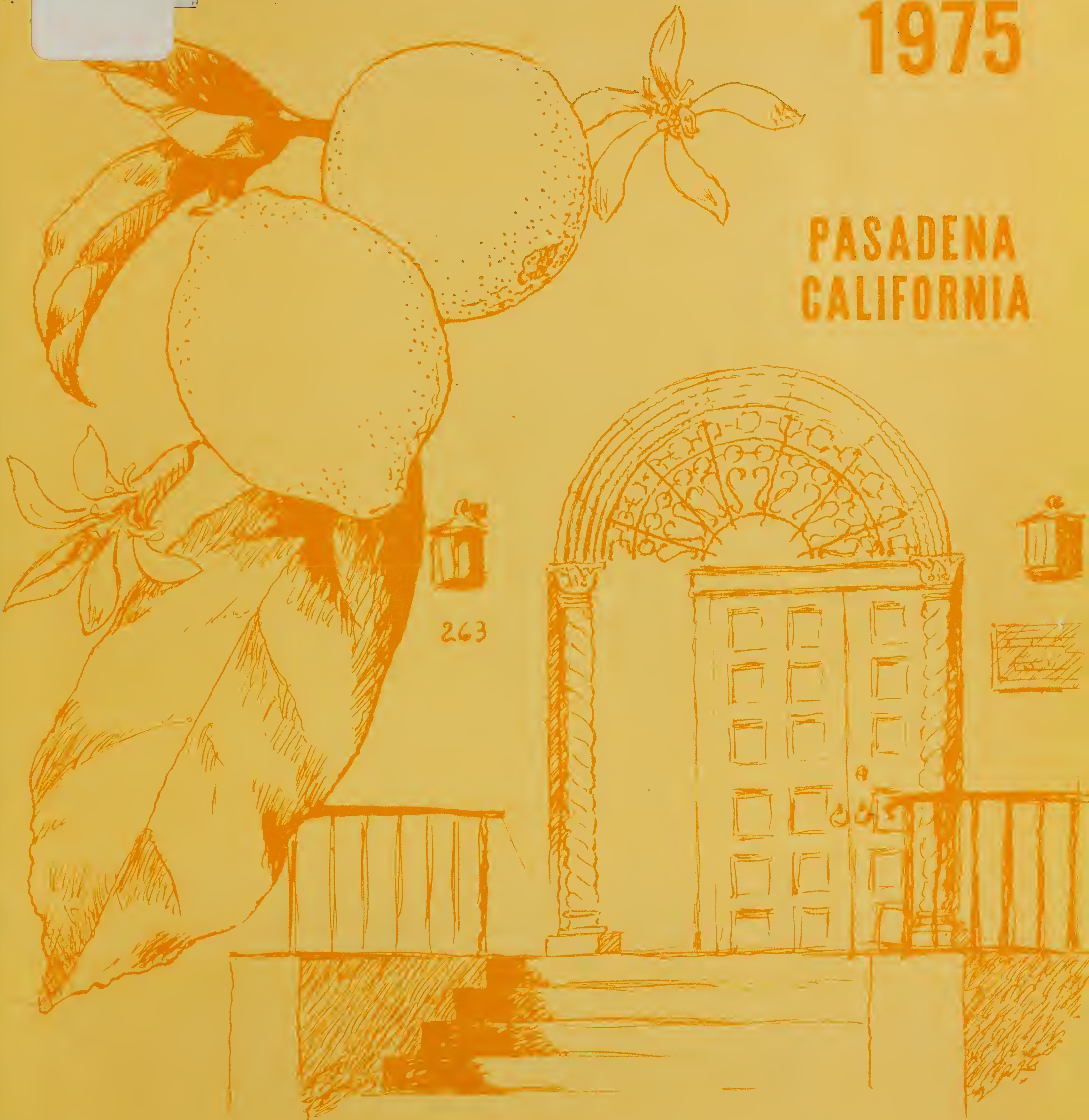
1975

PASADENA CALIFORNIA



CITRUS RESEARCH CONFERENCE

CITRUS RESEARCH CONFERENCE

December 16, 1975

Pasadena, California

ABSTRACTS OF PAPERS

Sponsored By:

Fruit and Vegetable Chemistry Laboratory
263 South Chester Avenue
Pasadena, California 91106

Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE

FOREWORD

This Citrus Research Conference is being held to bring to members of the citrus and allied industries in southern California and Arizona the latest results of research on the chemistry, pharmacology, and technology of citrus fruits and their products carried on by the Agricultural Research Service, U.S. Department of Agriculture. The following are participating in this year's conference:

Western Region

Fruit and Vegetable Chemistry Laboratory
263 South Chester Avenue, Pasadena, California 91106

Western Regional Research Center
Berkeley, California 94710

Southern Region

Citrus and Subtropical Products Laboratory
600 Avenue S, N.W., Winter Haven, Florida 33882

Conference headquarters:

Huntington-Sheraton Hotel
1401 South Oak Knoll Avenue
Pasadena, California 91109

P R O G R A M

CITRUS RESEARCH CONFERENCE

Tuesday, December 16, 1975

MORNING SESSION - 9:00 A.M. — Abstract on page

WELCOMING REMARKS: W. D. McClellan, Area Director, California-Hawaii-Nevada Area, ARS/USDA, Fresno, California

INTRODUCTORY REMARKS: V. P. Maier, Director, Fruit and Vegetable Chemistry Laboratory, Pasadena, California

ENHANCEMENT OF COLOR AND PROVITAMIN A QUALITY OF CITRUS FRUIT*
I. CURRENT STATUS OF THE WORK ON BIOREGULATORS

Henry Yokoyama, Wan-Jean Hsu, Stephen M. Poling, Charles DeBenedict and Ernest Hayman
Fruit and Vegetable Chemistry Laboratory
Pasadena, California

Studies were continued on the development of better bioregulators for improving the color and provitamin A content of citrus fruit, particularly oranges. Previous structure-activity studies showed that it is possible to devise bioregulators that cause buildup of carotenoids other than lycopene, the red pigment that lacks provitamin A activity. During the past year, emphasis was placed on the design and synthesis of bioregulators that influence the stimulation and accumulation of the preferred provitamin A carotenoid, beta-carotene. Beta-carotene also imparts to the fruit a highly desirable deep-orange coloration. Thus, any large buildup of beta-carotene, if lycopene buildup is low, will be reflected in coloration of the fruit, which never develops beyond the deep-orange stage. In this phase of our program, we are coming much closer to meeting the needs of the citrus industry. However, despite the progress being made in the design and synthesis of bioregulators that elicit more appropriate color and provitamin A responses, a number of problems still remain. The problem of penetration of the bioregulators into the endocarp is being actively studied. Except for the thinner skinned citrus like tangerines, surface application of bioregulators by dipping or spraying the fruit generally does not induce color response in the endocarp. Moreover, some of the bioregulators must be applied at relatively high concentrations (up to 5% w/v) to bring about rapid and uniform color response. Related areas of research that are being investigated include bioregulator-membrane interaction, correlation of bioregulatory effect with fruit maturity, and the potential synergistic effects amongst bioregulators and/or other agents. These studies are being conducted to attain better penetration and uniformity of color response at lower concentrations. At the more fundamental level, questions regarding the mode of action and the metabolic fate of the applied bioregulators need to be more fully answered.

In associated work on citrus byproducts, collaborative studies have been initiated with the products research group at Sunkist and the aquaculture group at the University of Rhode Island to find ways of utilizing orange peel as a pigment source for use

---

*Supported in part by the California Citrus Advisory Board and the Florida Citrus Commission.

in aquaculture feeds. The color of the flesh and skin of marine life used for food such as salmon, trout, seabream, and prawn is of prime economic importance and is due primarily to the deposition and/or modification of ingested carotenoid pigments. These marine animals do not have the capacity to biosynthesize carotenoid pigments; the pigments are derived from feed sources such as algae, diatoms, crustaceae, etc. In general, to be effective, the ingested carotenoids must be of the xanthophyll type, that is, they must possess oxygenated or hydroxyl functional groups. Thus, the flesh of a trout fed only non-carotenoid or hydrocarbon carotenoid containing feed is colorless. In contrast, the flesh of a xanthophyll-fed trout is deep orange. Preliminary feeding studies on sea trout were carried out at the University of Rhode Island using carotenoid pigment extracts from dehydrated orange peel supplied by Sunkist. Examination of the flesh pigments indicated the trout were capable of absorbing and depositing citrus xanthophylls. Additional preliminary investigations were conducted with carotenoid extracts from xanthophyll-enhanced orange peel. The results again indicated the absorption and deposition of citrus carotenoids in the flesh of the trout; moreover, no adverse effect from the applied bioregulator was evident. Thus, color-enhanced peel shows substantial promise as a feed supplement in aquaculture. Recently, we have demonstrated that color enhancement of citrus peel can be brought about by treating the peel after the fruit is juiced. Further studies on a much larger scale are currently underway.

II. NEW SYNTHETIC BIOREGULATORS OF CAROTENOID BIOSYNTHESIS

Stephen M. Poling, Wan-Jean Hsu and Henry Yokoyama
Fruit and Vegetable Chemistry Laboratory
Pasadena, California

We have previously reported on the ability of various tertiary amines to enhance the natural color and provitamin A content of citrus. These bioregulators stimulate the natural biosynthetic pathway causing an increased accumulation of the naturally occurring carotenoid pigments and, therefore, an enhanced color and provitamin A content.

The Hansch approach to correlating biological activity with molecular structure has been useful in understanding the ability of the previously synthesized bioregulators to stimulate biosynthesis. The Hansch approach, in its simplest form, takes into account two factors, the value of the logarithm of the octanol-water partition coefficient (log P) and the Hammett constants. The partition coefficient is the concentration of the compound in octanol divided by the concentration in water when the two phases are in contact and represents the relative affinity of the compound for lipids as compared to aqueous solutions. Log P will reflect the ability of the compound to pass through the various aqueous and lipid layers in the cell and, therefore, should bear a strong correlation to the concentration at the regulatory site. Previous results have shown the effectiveness of the test compounds increases with increasing values of log P up to about 4.5, at which point the compounds begin to cause some peel damage. The Hammett constants reflect the electron withdrawing or releasing ability of the substituent groups and are correlated to the strength of the interaction of the bioregulator with the active site. Past work indicates that strongly electron withdrawing substituents greatly increase the effectiveness of the bioregulators.

Previously tested compounds varied mostly in the hydrocarbon skeleton of the molecule, which gives a wide range of values for log P but contained only a few substituents with differing Hammett constants. This clearly showed the effect of log P but gave only limited results on the effect of the Hammett constants. A new series of compounds based on diethylaminoethyl benzoate (p-$RC_6H_4COOCH_2CH_2NEt_2$, R = H, NH_2, CN, NO_2, MeO, Me, tert-Bu, F, Cl, Br) gives a wide range of values of the Hammett constants as well as varying log P and clearly shows the increase in activity caused by electron withdrawing substituents. The series p-$RC_6H_4CH_2NEt_2$ (R = H, Me, NO_2) confirms this effect and shows that, in some cases, the increase in the Hammett constant causes a greater increase in the activity than increasing log P. The final series

of new compounds, $RC_6H_4OCH_2CH_2NEt_2$ (R = o-Me, m-Me, p-Me), shows the effects of varying the position of the substituent group. The para substituted compounds are the most active. There is a large decrease in activity when the substituent is placed at the meta position while the ortho substituted compound is almost completely inactive. These results confirm the earlier findings that increasing log P and more electron withdrawing substituents increase the activity of carotenoid bioregulators.

These new compounds were tested on Marsh seedless grapefruit. Examination of the flavedo of the treated fruit showed the response pattern was similar to that of the previously tested tertiary amines. The main effect was the accumulation of the red pigment, lycopene. Although all the compounds caused lycopene accumulation, the appearance of the fruit varied widely. In some cases, the fruit changed rapidly from yellow to red, in others the color change was slower and the fruit retained an orange color for extended periods. Some compounds caused the color to appear in large blotches while for other compounds the color is extremely uniform in appearance with little variation from fruit to fruit.

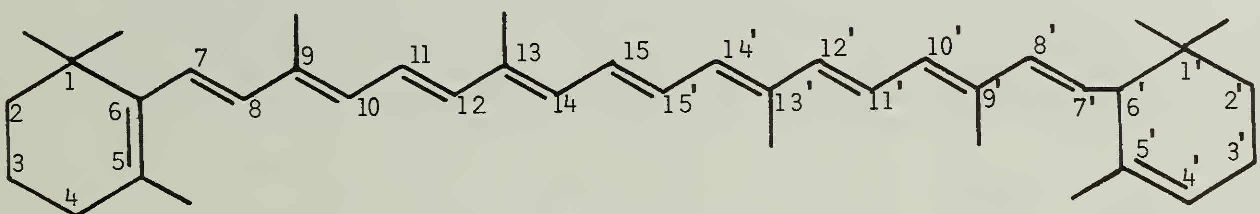
In addition to the elucidation of the structure-activity relationships of the bioregulators, several of the benzoates caused very significant increases in the provitamin A carotenoids. p-$BrC_6H_4COOCH_2CH_2NEt_2$ in particular caused a 25-fold increase in the provitamin A β-carotene as well as large increases in α- and γ-carotene.

These new compounds show a potential usefulness for increasing the nutritional level, especially some of the benzoates which caused much larger increases in the provitamin A carotenoids than previously seen, as well as in bringing about desirable color changes in citrus fruits.

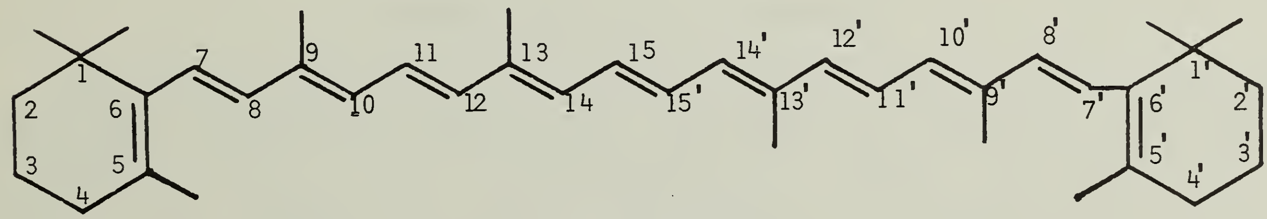
III. PRELIMINARY STUDIES ON THE CAROTENE XANTHOPHYLL RELATIONSHIP IN CITRUS

Wan-Jean Hsu, Stephen M. Poling and Henry Yokoyama
Fruit and Vegetable Chemistry Laboratory
Pasadena, California

Xanthophylls are a group of oxygen-containing carotenoids. The most commonly encountered xanthophylls in citrus fruits are those with a hydroxyl group at the 3 and/or 3' positions (e.g., lutein and zeaxanthin) and those with an epoxide group at the 5, 6 position (e.g., violaxanthin) of the ionone ring in the molecule of α- and/or β-carotene. It has long been agreed that the insertion of the hydroxyl and epoxide groups is a late step in the biosynthetic sequence, happening after the cyclization at both ends of the C_{40}-carotenoid molecule has been completed.



α-Carotene



β-Carotene

Group III bioregulators developed in our laboratory have been found to induce the formation of xanthophylls without large accumulation of lycopene and other hydrocarbon carotenoids. However, the induction process takes longer than two weeks at room temperature. The slowness of the induction process by this group of bioregulators might possibly be due to weak stimulation of the production of the xanthophyll precursors, α- and β-carotene. Higher levels of xanthophyll accumulation would be very useful in developing citrus peel as a pigment source for aquaculture feeds, as mentioned in an earlier paper.

In the past year, bioregulators which will cause a large increase in the provitamin A carotenoid content of citrus fruit were designed and synthesized. As mentioned in the preceding paper, up to a 25-fold increase in β-carotene was observed in the case of N,N-diethylaminoethyl-p-bromobenzoate (p-$BrC_6H_4COOCH_2CH_2NEt_2$). Fruits respond to the β-carotene inducing bioregulators very well. Accumulation of β-carotene starts within four days and continues thereafter. Because of the fast acting ability of this group of bioregulators, and the aforementioned relationship between the carotenes and xanthophylls, an experiment was carried out to see whether the immediate increase in β-carotene caused by the β-carotene inducer could be channeled into increased xanthophyll accumulation under the simultaneous influence of the xanthophyll inducer. Results on Marsh white grapefruit showed that β-carotene content started to increase at an early stage after treatment and continued to increase thereafter, while xanthophyll accumulation was not affected in the early stages and only increased later. The treated fruit accumulated large amounts of β-carotene and smaller amounts of the xanthophyll carotenoids, just as if the fruit had been treated with these two bioregulators separately. If xanthophylls do arise in citrus fruit by the biosynthetic sequence mentioned earlier, these results are unexpected. Further studies of this type are underway to attempt stimulation of xanthophyll accumulation in citrus, and, in so doing, to explore the biosynthetic pathway.

ASSESSING POTENTIAL EFFECTS OF MECHANICAL HARVESTING ON CITRUS PROCESS TECHNOLOGY

Robert E. Berry, William L. Bryan
and Manuel G. Moshonas
Citrus and Subtropical Products Laboratory
Winter Haven, Florida

The Florida citrus industry is moving toward mechanical harvesting of oranges for processed products. Anticipated expansion of mechanical harvesting may bring about several changes in processing requirements. Fruit arriving at the processing plant will contain larger quantities of trash (twigs, leaves, sand, etc.) and, probably, larger amounts of bruised, damaged, unwholesome or unstable fruit. Improved mechanization of unloading facilities will be required to effectively separate trash and unwholesome fruit at the increased rates.

In most mechanical harvesting systems being tested, abscission chemicals will be required to loosen the fruit prior to harvesting. These chemicals function by altering metabolic pathways, bringing about release of wound ethylene, and might thus affect formation or balance of flavor components. They could also affect storage stability of the fruit in bins and could even have a potential effect on quality or storage stability of products. Since their principal effect is on the peel, they could also cause changes in peel oil or other peel-related byproducts.

Studies are underway at the U.S. Citrus and Subtropical Products Laboratory, Winter Haven, Florida, covering both these areas, and progress of these studies will be reviewed.

Mechanical Fruit Grading

A pilot receiving line has been installed at a Florida plant to develop economical mechanical methods for removing trash and unwholesome fruit while unloading and conveying to storage bins at commercial rates (up to 60 boxes/min). The pilot line includes: 1) A system of belts, elevators and chutes for conveying. 2) An experimental destemmer designed to grasp and remove attached stems longer than 2" (loaned by a commercial company). 3) A 14-brush scrubber. 4) A mechanical grader designed to remove the most degraded, unwholesome fruit and less than 20% of the sound fruit.

The grader consists of a roller conveyer to align fruit into lanes, an acceleration ramp at 65°, a rotating drum where fruit strikes the surface at an angle and a belt conveyer with partitions to separate fruit by distance projected. Sound fruit are projected farther than unwholesome and conveyed from a separate stream.

A rotating drum has replaced the bounce pad or arc used in earlier models. Advantages of the rotating drum are: Better control on projection distance of fruit with a more narrow range of projection distances. Fruit can be graded at higher rates without collisions. The bounce surface can be continuously cleaned.

The pilot line was operated on trial runs with the following results: 1) Trash averaged 0.20% of each load with an average distribution of 42% leaves, 35% twigs and stems and 23% sand. 2) Fruit with attached stems varied from 2-9% and more than 50% of the stems over 2" long were removed. 3) Unwholesome fruit varied among loads from 1-6%. About half the unwholesome fruit, including all the most degraded and decomposed, fell into the "cull" stream with about 5% of the sound fruit. Sound fruit could be removed from this stream by manual grading or a second stage of mechanized grading.

Flavor Effects of Abscission Agents

Four abscission chemicals most commonly used in experimental and limited commercial trials are agents with the trade names Acti Aid, Release, Pik-Off, and Ethephon. The first three cause peel damage, releasing wound ethylene to promote abscission. Release and Pik-off are effective for Valencia oranges without damaging young fruit. Ethephon releases ethylene by chemical breakdown without damaging peel.

Canned pasteurized single-strength orange juices (SSOJ) were prepared from Hamlin, Pineapple and Valencia oranges and compared for flavor differences by triangle comparisons and for flavor preferences by paired comparisons. The following tests were carried out: 1) SSOJ from abscission treated experimental juices vs. non-treated controls; 2) experimental concentrates vs. controls; 3) stored experimental and control juices held at 70° and 85°F versus reference juices held at 0°F.

Experimental SSOJ from all cultivars and all treatments was distinguished from non-treated samples by a trained experienced taste panel. Many panel members indicated experimental juices had over-ripe flavor considered to be adverse. In most tests, the panel preferred the control juice but in a few cases there was no preference. However, in no test was the experimental juice preferred. Since these results were tested for detectable difference by a trained panel, it is uncertain whether general consumers would detect these flavor changes or whether such threshold flavor changes would be objectionable. Also, at current useage, mechanically harvested fruit is such a small proportion of total processed fruit, with which it would be blended, that flavor effects on current products would be negligible. Some pilot consumer tests with untrained panelists are being conducted.

Studies on composition of peel oil indicate small but definite differences in oil from abscission-treated fruit. Several components of the oil were lacking, and one or two previously un-

reported components were observed. These studies with mechanical grading and flavor affects of abscission agents emphasize the importance of carefully controlling concentration of abscission chemicals, utilizing minimum time for fruit loosening after spraying in order to minimize flavor changes, increasing rates of fruit handling to minimize flavor deterioration, and minimizing time fruit would be stored in bins before processing to ensure high-quality products and byproducts.

THE NATURE OF HESPERIDIN IN LEMON JUICE CLOUD*

Ronald E. Schuster and Raymond D. Bennett
Fruit and Vegetable Chemistry Laboratory
Pasadena, California

Hesperidin in the cloud of lemon juice concentrate is mainly in the form of crystalline needles, from 1 to 5 µm in length. Except for some crystals found adhering to pulp particles, they are present as individual crystals rather than aggregates. If juice is filtered immediately after extraction, hesperidin does not crystallize from the filtrate, and the hesperidin content of the cloud, determined by fluorescence spectroscopy, does not change during the concentration process. This indicates that the crystals are not formed by crystallization of soluble hesperidin from juice during extraction or processing.

The hesperidin content of cloud obtained from samples of commercial concentrates ranged from 21-54 mg/100 ml of juice, representing 8-19% by weight of the total cloud. This is much higher than the 0.4 mg/100 ml obtained from cloud of fresh hand reamed juice prepared in the laboratory. This is due to the fact that most of the hesperidin is found in the albedo portion of the fruit, which is not disrupted under normal laboratory extraction conditions.

When hesperidin was allowed to crystallize from water, aqueous solutions of different juice components, or juice serum, various types of crystals were formed, but in no case were they like those normally found in lemon juice cloud. Crystals of the latter type could be produced by adding hesperidin in an organic solvent to hot water and sonicating briefly. In water, these crystals had a strong tendency to form aggregates, but if they were isolated by centrifuging and then dispersed in juice serum by sonicating, a suspension of single crystals was formed. The light scattering efficiency (optical density at 600 nm/mg) of this model suspension was about the same as that of whole cloud. However, suspensions of crystals of the same length but several times thicker were much more turbid.

The solubility of hesperidin in water at the normal pH of lemon juice (2.3-2.4) is approximately 2 mg/100 ml. However, single strength serum from a lemon juice concentrate could keep about 15 mg/100 ml of added hesperidin in solution for an extended period of time. Since this juice serum already contained several mg/100 ml of hesperidin, the total solubility of the latter was about 10 times as great in serum as in water. In an attempt to isolate the component or components in lemon juice serum, which are responsible for this large increase in hesperidin solu-

*Work supported in part by the Citrus Products Technical Committee.

bility, we have carried out a number of experiments utilizing an apparatus employing synthetic membranes of various pore sizes. These membranes allow the separation of materials on the basis of their molecular size and shape. Single strength juice serum was diafiltered through a membrane with a molecular weight cut-off value of 10,000. The fraction which did not pass through was then diafiltered through a different membrane, which had a cut-off value of 10,000 for spherical molecules but was permeable to linear molecules up to about 100,000. In this case, the fraction which passed through the membrane was collected. The material isolated in this manner represented only 3% of the original soluble solids of the juice, but it retained the ability to solubilize hesperidin. Based upon the isolation procedure, the hesperidin-solubilizing factor appears to be a linear polymer with a molecular weight between 10,000 and 100,000. Approaches being used to further purify and characterize this material will be discussed.

MICROBIOLOGICAL ASSAY FOR JUICE CONTENT IN ORANGE JUICE BEVERAGES

Carl E. Vandercook and Dora C. Smolensky
Fruit and Vegetable Chemistry Laboratory
Pasadena, California

The problem of detecting adulteration in orange juice or of determining juice content in an orange juice product has attracted a great deal of interest over the years. Many constituents have been proposed as indicies of authenticity, however, no one seems to have used microbiological assay procedures to measure the content of orange juice in orange drinks. Microbiological assays are widely used for specific constituents such as vitamins and amino acids. In this investigation the concept of a microbiological assay based on a growth-limiting nutrient has been applied to the determination of the amount of an agricultural commodity in a processed food product. Presumably, one or more of the minor nutrients in the juice become growth limiting for the organism.

Briefly, the assay consists of the following steps: (1) sample preparation (clarifying by centrifugation, adjusting the pH to 6.5, and diluting to 20% juice); (2) preparation of inoculum; (3) inoculation of sterilized samples; (4) incubation for 30 hours at 37°C; and (5) measurement of cell growth as turbidity. Under the standard assay conditions, Lactobacillus plantarum was found to grow in proportion to the amount of orange juice in the sample. Imitation orange beverages did not support growth. Growth was also independent of normal ingredients in beverages such as sugars, acids, thickeners, coloring and flavoring agents.

Various steps in the experimental procedure which could introduce error were individually tested. The assay is relatively tolerant of minor variations, however, cumulative effects might introduce a greater error. The precision of the analyses, as shown by standard error, was around ± 0.002 absorbance units (AU) for replicates within a given experiment and ± 0.016 AU between replicate experiments in which the average turbidity was 0.330 AU.

Orange juices from various sources were assayed by this technique. The variance between samples compared very favorably with that of the chemical analyses for juice content performed in this laboratory. The results of the assay for orange juice using L. plantarum and several other bacteria will be presented. Application of the assay will be discussed along with the use of the previously reported chemical and statistical procedures.

ACID AND ESTER DERIVATIVES OF DIHYDROCHALCONE SWEETENERS

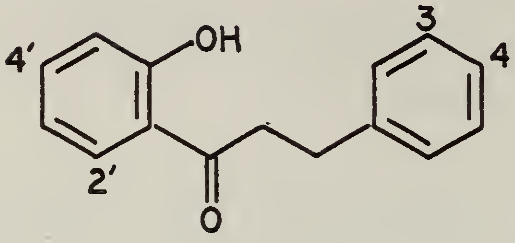
R. M. Horowitz and Bruno Gentili
Fruit and Vegetable Chemistry Laboratory
Pasadena, California

The bitter citrus flavanones naringin and neohesperidin yield, on reduction in alkali, the intensely sweet compounds naringin dihydrochalcone and neohesperidin dihydrochalcone. Since their discovery in 1961, many modifications of these dihydrochalcones have been made in order to clarify structure-activity relations and obtain compounds with improved taste or solubility. An ideal sweetener would be similar to sucrose in taste quality, would have adequate solubility and stability, and would be non-toxic, non-cariogenic and economically competitive.

To try to reach some of these goals, we have synthesized a series of acid and ester derivatives containing the $-OCH_2COOH$ or $-OCH_2COOCH_3$ groups. The structure and taste of some of these compounds are shown in the Table. Syntheses were accomplished by alkylating the appropriate flavanone, phloracetophenone or aldehyde derivative with methyl bromoacetate, followed by conversion of the alkylated intermediate to the dihydrochalcone. In certain cases, the desired compound was obtained by direct alkylation of the dihydrochalcone. The structure of most products was proved by the method of synthesis, and it was further confirmed by ultraviolet and nuclear magnetic resonance data. Satisfactory elemental analyses were obtained for all compounds.

Structure-activity relations and practical aspects of the compounds in this series will be discussed. Critical factors for taste are (a) the location of the $-OCH_2COOR$ group and (b) whether the group is in the form of an ester or free acid.

EFFECT OF ACID OR ESTER SUBSTITUENTS ON TASTE



Substituent at				
2'	4'	3	4	Taste
OH	Neo-O		OCH_2COOMe	-++
OH	Neo-O		OCH_2COOH	Nil
OH	Neo-O	OH	OCH_2COOMe	+++
OH	Neo-O	OH	OCH_2COOH	Nil
OH	Neo-O	OMe	OCH_2COOMe	-
OH	Neo-O	OMe	OCH_2COOH	Nil
OCH_2COOMe	Neo-O		OH	-
OCH_2COOH	Neo-O		OH	Nil
OCH_2COOMe	Neo-O	OH	OMe	++
OCH_2COOH	Neo-O	OH	OMe	Nil
	OCH_2COOMe	OH	OMe	Nil
	OCH_2COOH	OH	OMe	+
OH	OCH_2COOMe	OH	OMe	Nil
OH	OCH_2COOH	OH	OMe	+++
OCH_2COOMe	OCH_2COOMe	OH	OMe	Nil
OCH_2COOH	OCH_2COOH	OH	OMe	+

+ = sweet; - = bitter; Neo = β-Neohesperidosyl

LONG-TERM TOXICITY STUDIES FOR THE SAFETY EVALUATION OF NEOHESPERIDIN DIHYDROCHALCONE SWEETENER

M. R. Gumbmann, D. H. Gould, D. J. Robbins and A. N. Booth
Pharmacology Research Unit
Western Regional Research Center
Berkeley, California

As debate continues over the safety of low-calorie sweeteners, such as cyclamate, aspartame and saccharin, the demand for new sweeteners to permit some flexibility of choice in specific applications steadily grows. However, most promising new sweeteners are at an early stage of development and a long way from market. There is particular interest in the dihydrochalcone sweeteners at this time, as toxicity studies on neohesperidin dihydrochalcone (NDHC) near completion.

Safety evaluation of new food additives is a lengthy and costly process, beginning with tests in laboratory animals of acute toxicity and short-term, sub-acute studies to define the nature of toxic effects and their relationship to dose. Studies such as these, of NDHC in rats, were started in 1965 and continued through 1968. They indicated no adverse effects from levels up to 5% NDHC in the diet.

Long-term feeding studies are conducted primarily to detect carcinogenesis but include multigeneration reproduction and teratology. Such studies, covering significant portions of the normal life-span of rats, were completed in 1973. Test animals were fed NDHC for two years at levels up to 5% of the diet and, in another group of rats, for one year at 10%. Adverse effects consisted of less body weight gain and greater liver, kidney, and thyroid weights, compared to controls, in both sexes fed 5% NDHC for two years. However, supplementation of the basal diet with additional vitamins and minerals counteracted these differences. No abnormalities related to ingestion of NDHC were detected (a) in analyses of blood and urine and (b) in the three generation reproduction study, including tests for fetal malformations. Among the most important criteria of toxicity is histopathological evaluation of tissues, which takes place at the end of a study. The only histological finding related to consumption of sweetener was an increased incidence of focal renal cortical atrophy in the kidneys of female rats. However, upon modification of the basal diet, as mentioned above, this lesion was not observed.

A two-year feeding study in dogs is scheduled for completion in January 1976. Daily, the dogs have received levels of NDHC up to 2 g per kg body weight, which is equivalent to approximately 7% of their diet. Blood and urine analyses and physical examination at six-month intervals have revealed no apparent adverse effects related to ingestion of NDHC.

BIOCHEMISTRY OF LIMONOIDS*

Shin Hasegawa and Kyung S. Kim
Fruit and Vegetable Chemistry Laboratory
Pasadena, California

A New Limonin-debittering Enzyme

Our continuing search for microbial enzymes which degrade limonoids has resulted in the isolation of a species of bacterium which produces a new type of intracellular limonoate dehydrogenase. The bacterium, which was isolated from soil and designated No. 342-152-1, metabolizes limonoate through two pathways: one through 17-dehydrolimonoate and the other through deoxylimonin, in a ratio of 3 to 1.

When cell-free extracts of the organism were fractionated on a DEAE-cellulose column, we observed, in addition to limonoate dehydrogenase activity, an active fraction which was capable of reducing NAD without the addition of a substrate. Further investigation revealed that the limonoate dehydrogenase of No. 342-152-1, which was originally thought to be a single form of the enzyme, was more complicated in that the enzyme could firmly bind with limonoate, forming a stable complex. This complex functions in the presence of cofactor like other limonoate dehydrogenases which catalyze the conversion of limonoate to 17-dehydrolimonoate. However, it has characteristics quite different from those of the limonoate dehydrogenases previously reported.

The complex has unusual activity at different hydrogen ion concentrations; it has optimal activity at pH 8.5 and 9.5, and it has very low activity at pH 9.0. The complex is much more stable than the free enzyme. About a 20° C higher temperature is necessary to inactivate the complex. It is of interest that the complex is very stable at pH 9.0. Both the complex and the free form require NAD as a cofactor and need sulfhydryl groups and Zn^{++} ions for their activities. The molecular weight of the complex was estimated to be 260,000, using Sephadex G-200. The possible application of this limonoate dehydrogenase complex will be discussed.

Biosynthesis of ^{14}C-labelled Limonin

Although substantial progress has been made in studying the bacterial degradation of limonoids during the past five years, little is known about the biochemistry of limonoids in citrus. Attempts to follow the biological changes in limonoid molecules in citrus have met with difficulties because the net change is low. The use of radioactively labelled limonoids could be an excellent approach to biogenesis and biodegradation of limonoids in citrus.

*Work supported in part by the Citrus Products Technical Committee.

We recently observed that limonoids were present in fruit right after the blossom dropped, and the content increased as the fruit grew. We also observed that limonoids were actively synthesized in young citrus leaves. For instance, 10-mg size lemon leaves contained over 2000 ppm limonin (which appeared to be present as limonoate A-ring lactone). The limonin content per leaf increased as the leaves grew. However, mature leaves contained only trace amounts of limonoids.

These results suggested that young leaves and fruits would be excellent tissues for use in study of the formation of limonoids and in the preparation of labelled limonoids. Na acetate-2-^{14}C fed to leaves adjacent to fruit on young navel orange trees (5 g size) was incorporated into the limonin molecules of the fruit at low but useful levels. The use of labelled mevalonic acid in place of acetate should increase the amount of ^{14}C incorporated.

BIOSYNTHESIS AND METABOLISM OF LIMONIN: POSSIBLE PATHWAYS BASED UPON STRUCTURES OF MINOR LIMONOIDS

Raymond D. Bennett
Fruit and Vegetable Chemistry Laboratory
Pasadena, California

When the structures of the bitter principle limonin and the other three major citrus limonoids became known, a biosynthetic pathway was proposed: deacetylnomilin $\longrightarrow$ nomilin $\longrightarrow$ obacunone $\longrightarrow$ limonin. However, this sequence required other intermediates which had not been isolated from citrus fruits. This led us to undertake an investigation of minor citrus limonoids. During the course of this work, we have isolated several limonoids which may be involved in limonin biosynthesis, and also some other compounds which appear to be metabolites of limonin.

If obacunone is a precursor of limonin, obacunoic acid and isoobacunoic acid are likely intermediates. These compounds had been prepared chemically from obacunone, but at the start of this work neither was known to occur in citrus. We found isoobacunoic acid in grapefruit seeds, but we were unable to detect obacunoic acid. However, we also found two new compounds, nomilinic acid and deacetylnomilinic acid. The latter could be a general precursor of limonoids, producing nomilinic acid by acetylation, deacetylnomilin by lactone ring closure, or isoobacunoic acid by ether ring closure. Isoobacunoic acid could then be converted to limonin by hydroxylation and lactone ring closure.

Another pathway could involve ichangin as an immediate precursor of limonin. Ichangin was originally isolated from the rare Ichang lemon, but we have since found it in grapefruit seeds and lemon seeds. Ichangin could be formed from deacetylnomilinic acid by hydroxylation and lactone ring closure, and it could be converted to limonin by ether ring closure. This pathway or the one proceeding from deacetylnomilinic acid via isoobacunoic acid now seem more likely than the one originally postulated, based upon our failure to detect obacunoic acid and the relatively large amounts of obacunone and nomilin which accompany limonin. Biosynthetic intermediates are usually turned over rapidly and, therefore, are found in low concentrations.

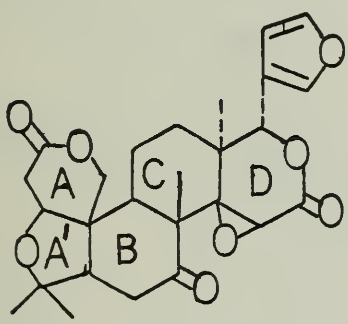
The work of Hasegawa, at this laboratory, on bacterial metabolism of limonoids led to the discovery of two metabolic pathways. One involved oxidation at C-17 of limonoic acid A-ring lactone (the naturally occurring form of limonin in fruit tissues) to 17-dehydrolimonoic acid A-ring lactone, and the other proceeded by reduction of limonin to deoxylimonin, followed by cleavage of the B-ring to produce deoxylimonic acid. Both 17-dehydrolimonoic acid A-ring lactone and deoxylimonic acid have now been isolated from citrus. Deoxylimonin had been known as a

citrus constituent prior to the microbiological work, but it was considered to be a side product of limonin biosynthesis rather than a metabolite. Our finding of deoxylimonic acid now makes it likely that, as in bacteria, deoxylimonin is the first step in a metabolic pathway.

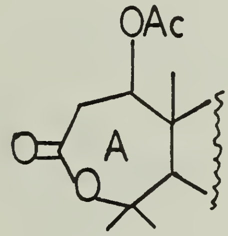
A third possible metabolic sequence is suggested by our finding of a new limonoid, which we have named isolimonic acid to indicate its relationship to deoxylimonic acid. This compound could be derived by oxidative cleavage of the B-ring of limonin. In contrast to deoxylimonic acid, the epoxide group has remained intact.

We have isolated two limonoids, limonol and obacunol, in which the 7-carbonyl has been reduced to a 7α-alcohol. This suggests another possible metabolic pathway, since limonol (which had previously been prepared by chemical reduction of limonin but was not known as a natural product) on treatment with base undergoes rearrangement and loss of the furan ring to produce merolimonol. Only limonoids containing a 7α-hydroxyl group react in this way. Another new citrus limonoid, deoxylimonol, combines this structural feature with loss of the epoxide group associated with the deoxylimonic acid pathway.

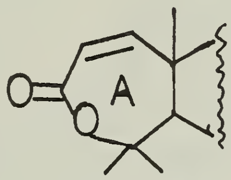
The results of this work have suggested several plausible pathways for the biosynthesis and metabolism of limonin. To find out which are actually operative in citrus fruits, preparation and administration of radioactively labelled limonoids will be necessary.



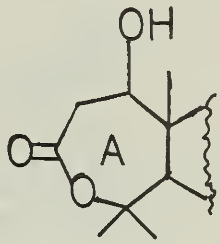
Limonin



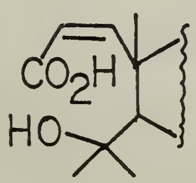
Nomilin



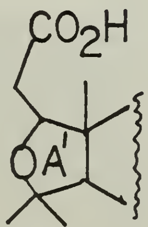
Obacunone



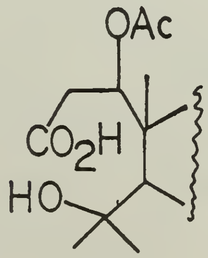
Deacetylnomilin



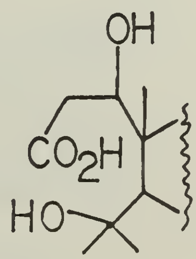
Obacunoic Acid



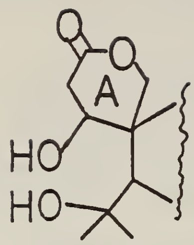
Isoobacunoic Acid



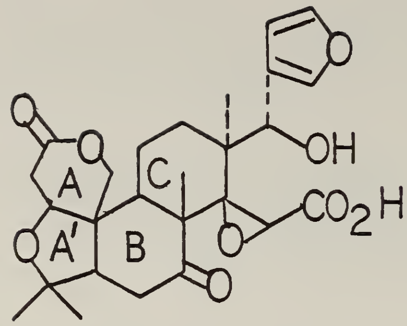
Nomilinic Acid



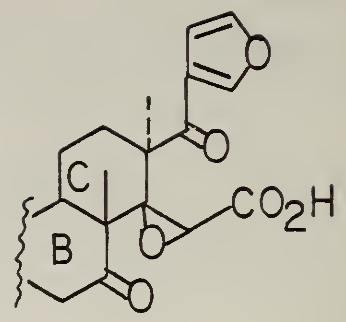
Deacetylnomilinic Acid



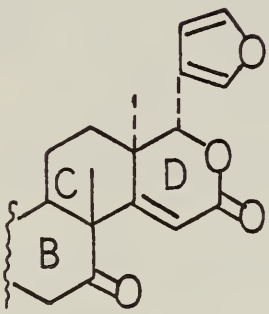
Ichangin



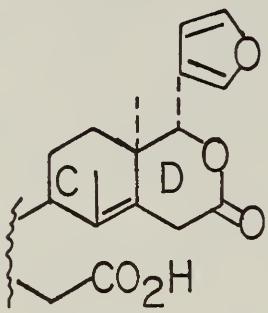
Limonoic Acid
A-Ring Lactone



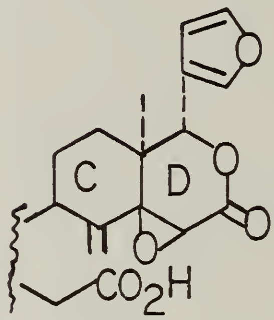
17-Dehydrolimonoic Acid
A-Ring Lactone



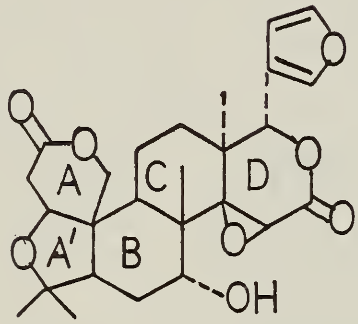
Deoxylimonin



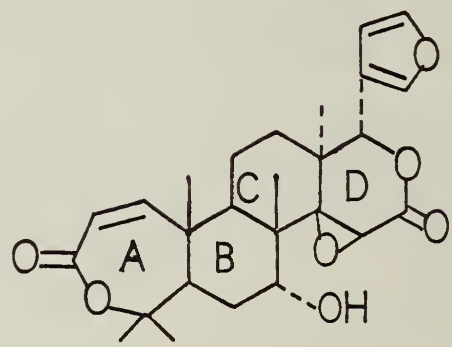
Deoxylimonic
Acid



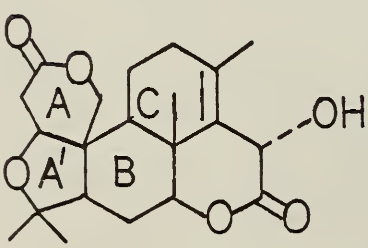
Isolimonic
Acid



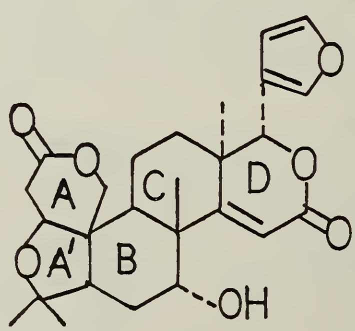
Limonol



Obacunol



Merolimonol



Deoxylimonol

LIST OF PUBLICATIONS AND PATENTS*

WESTERN REGION

Fruit and Vegetable Chemistry Laboratory
263 South Chester Avenue, Pasadena, California 91106

METABOLISM OF LIMONOIDS, ISOLATION AND CHARACTERIZATION OF DEOXYLIMONIN HYDROLASE FROM *PSEUDOMONAS*
Shin Hasegawa, V. P. Maier, S. N. Border and R. D. Bennett
J. Agr. Food Chem. 22, 1093-1096 (1974).

SOME FACTORS AFFECTING SENSORY THRESHOLDS AND RELATIVE BITTERNESS OF LIMONIN AND NARINGIN
D. Guadagni, V. P. Maier and J. H. Turnbaugh
J. Sci. Fd. Agr. 25, 1199-1205 (1974).

EFFECT OF SUBTHRESHOLD CONCENTRATIONS OF LIMONIN, NARINGIN AND SWEETENERS ON BITTERNESS PERCEPTION
D. Guadagni, V. P. Maier and J. H. Turnbaugh
J. Sci. Fd. Agr. 25, 1349-1354 (1974).

APIGENIN 4'-O-β-D-GLUCOSIDE 7-O-β-D-GLUCURONIDE: THE COPIGMENT IN THE BLUE PIGMENT OF *CENTAUREA CYANUS*
S. Asen and R. M. Horowitz
Phytochem. 13, 1219-1223 (1974).

CAROTENOID BIOSYNTHESIS IN *RHODTORULA GLUTINIS*
Ernest P. Hayman and Henry Yokoyama
J. of Bacteriol. 120, 1339-1343 (1974).

MULTIPLE AUTOMATED ANALYSES FOR ORANGE JUICE CONTENT: DETERMINATION OF TOTAL SUGARS, REDUCING SUGARS, TOTAL ACIDITY, TOTAL AMINO ACIDS, AND PHENOLICS
Carl E. Vandercook, Ruth L. Price, Christina A. Harrington
J. Assoc. Off. Anal. Chem. 58, 482-487 (1975).

LIMONOATE DEHYDROGENASE AND PREPARATION THEREOF
Shin Hasegawa and Linda C. Brewster
U.S. Patent No. 3,869,345. Patented March 4, 1975.

DIHYDROCHALCONE GALACTOSIDES AND THEIR USE AS SWEETENING AGENTS
Robert M. Horowitz and Bruno Gentili
U.S. Patent No. 3,876,777. Patented April 8, 1975.

DIHYDROCHALCONE GALACTOSIDES AND THEIR USE AS SWEETENING AGENTS
Robert M. Horowitz and Bruno Gentili
U.S. Patent No. 3,890,296. Patented June 17, 1975.

---

*Reprints are available at the addresses indicated; patents are available only by purchase at 50¢ a copy from the U.S. Patent Office, Washington, D.C. 20231.

METHOD OF ENHANCING COLORATION OF FRUITS AND VEGETABLES WITH A DIALHYLAMINO-ALKOXYBENZENE
Henry Yokoyama, Wan-Jean Hsu and Stephen Poling
U.S. Patent No. 3,864,501. Patented February 4, 1975.

CHEMICAL INDUCERS OF CAROTENOGENESIS
Wan-Jean Hsu, Stephen M. Poling, Charles DeBenedict, Charles Rudash and Henry Yokoyama
J. Agr. Food Chem. 23, 831-834 (1975).

SOUTHERN REGION

Citrus and Subtropical Products Laboratory
600 Avenue S, N.W., Winter Haven, Florida 33880

QUANTITATIVE COMPOSITON OF COLD-PRESSED ORANGE OILS
Philip E. Shaw and Richard L. Coleman
J. Agr. Food Chem. 22, 785-787 (1974).

RELATIONSHIP OF FURFURAL TO TEMPERATURE ABUSE AND FLAVOR CHANGE IN COMMERCIALLY CANNED SINGLE-STRENGTH ORANGE JUICE
Steven Nagy and Howard L. Dinsmore
J. Food Sci. 39, 1116-1119 (1974).

FATTY ACIDS OF TRIGLYCERIDES AND STEROL ESTERS FROM DUNCAN GRAPEFRUIT, DANCY MANDARIN AND THEIR TANGELO HYDRIDS
Harold E. Nordby and Steven Nagy
Phytochem. 13, 2215-2218 (1974).

ULTRAVIOLET ABSORPTION METHOD FOR EVALUATING CITRUS ESSENCES
J. M. Randall, W. L. Bryan, O. W. Bissett and R. E. Berry
J. Food Sci. 38, 1047-1050 (1973).

ASCORBIC ACID RETENTION IN ORANGE JUICE AS RELATED TO CONTAINER TYPE
Owen W. Bissett and Robert E. Berry
J. Food Sci. 40, 178-180 (1975).

ESTIMATION OF ASCORBIC ACID IN ORANGE JUICE BY A CHRONOMETRIC METHOD
Bongwoo Roe and Joseph H. Bruemmer
Proc. Fla. State Hort. Soc. 87, 210-213 (1974).

ANALYSIS OF CONCENTRATED ORANGE ESSENCE AND COMPARISON WITH KNOWN ESSENCE COMPOSITION
Philip E. Shaw and Manuel G. Moshonas
Proc. Fla. State Hort. Soc. 87, 305-310 (1974).

MECHANICAL GRADING OF ORANGES BASED ON DYNAMIC BEHAVIOR
William L. Bryan, Barry J. Anderson and Gary L. Norman
Proc. Fla. State Hort. Soc. 87, 313-318 (1974).

CHARACTERIZATION OF CITRUS CULTIVARS AND SEPARATION OF NUCELLAR AND ZYGOTIC SEEDLINGS BY THIN LAYER CHROMATOGRAPHY
James H. Tatum, Robert E. Berry and C. Jack Hearn
Proc. Fla. State Hort. Soc. 87, 75-81 (1974).

THE RELATIONSHIP OF LONG-CHAIN HYDROCARBONS TO THE CHEMOTAXONOMY OF CI
Harold E. Nordby and Steven Nagy
Proc. Fla. State Hort. Soc. 87, 70-74 (1974).

BIOCHEMICAL CHANGES IN GRAPEFRUIT STORED IN AIR CONTAINING ETHYLENE
Paul L. Davis, Bongwoo Roe and Joseph H. Bruemmer
Proc. Fla. State Hort. Soc. 87, 222-227 (1974).

LIPID COMPOSITION OF COMMERCIALLY CANNED SINGLE-STRENGTH ORANGE JUICE
Steven Nagy, Harold E. Nordby and John M. Smoot
J. Am. Oil Chem. Soc. 52, 121-123 (1975).

SATURATED AND MONO-UNSATURATED LONG-CHAIN HYDROCARBON PROFILES FROM CITRUS UNSHIU JUICE SACS
Harold E. Nordby and Steven Nagy
Phytochem. 14, 183-187 (1975).

DEGRADATION PRODUCTS FORMED IN CANNED SINGLE-STRENGTH ORANGE JUICE DURING STORAGE
James H. Tatum, Steven Nagy and Robert E. Berry
J. Food Sci. 40, 707-709 (1975).

PERESTER OXIDATION OF (+)-LIMONENE TO (-)-CARVONE AND PIPERITENONE
Charles W. Wilson and Philip E. Shaw
J. Agr. Food Chem. 23, 636-638 (1975).

TWO SIMPLE METHODS FOR EVALUATING CITRUS ESSENCE: BROMATE TITRATION AND GAS CHROMATOGRAPHY
Manuel G. Moshonas and Philip E. Shaw
Int. Flavours 6, 133-134 (1975).

COMPOSITION AND FLAVOUR EVALUATION OF A VOLATILE FRACTION FROM A COLD-PRESSED VALENCIA ORANGE OIL
Philip E. Shaw and Richard L. Coleman
Int. Flavours 6, 190 (1975).

Food Crops Utilization Unit
P.O. Box 388, Weslaco, Texas 78596

BLENDING TO IMPROVE QUALITY OF TEXAS ORANGE JUICE PRODUCTS
Robert R. Cruse and Bruce J. Lime
J. Rio Grande Valley Hort. Soc. 28, 154-163 (1974).

Trade names are used in this publication solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement by the Department over other products not mentioned.

GPO 967-316